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# Identification and Characterization of Two Unusual cGMP-stimulated Phoshodiesterases in *Dictyostelium*

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Recently, we recognized two genes, *gbpA* and *gbpB*, encoding putative cGMP-binding proteins with a $Zn^{2+}$-hydrolase domain and two cyclic nucleotide binding domains. The $Zn^{2+}$-hydrolase domains belong to the superfamily of β-lactamases, also harboring a small family of class II phosphodiesterases from bacteria and lower eukaryotes. Gene inactivation and overexpression studies demonstrate that *gbpA* encodes the cGMP-stimulated cGMP-phosphodiesterase that was characterized biochemically previously and was shown to be involved in chemotaxis. cAMP neither activates nor is a substrate of GbpA. The *gbpB* gene is expressed mainly in the multicellular stage and seems to encode a dual specificity phosphodiesterase with preference for cAMP. The enzyme hydrolyses cAMP ~9-fold faster than cGMP and is activated by cAMP and cGMP with a $K_A$ value of ~0.7 and 2.3 μM, respectively. Cells with a deletion of the *gbpB* gene have increased basal and receptor stimulated cAMP levels and are sporogeneous. We propose that GbpA and GbpB hydrolyze the substrate in the $Zn^{2+}$-hydrolase domain, whereas the cyclic nucleotide binding domains mediate activation. The human cGMP-stimulated cAMP/cGMP phosphodiesterase has similar biochemical properties, but a completely different topology: hydrolysis takes place by a class I catalytic domain and GAF domains mediate cGMP activation.

## INTRODUCTION

cAMP and cGMP are important signaling molecules in prokaryotes and eukaryotes. These molecules are produced by cyclases, degraded by phosphodiesterases, and exert their functions by binding to specific proteins. In prokaryotes, cAMP regulates gene expression via binding to the cyclic nucleotide binding (cNB) domain of catabolic repressor transcription factors (Passner *et al.*, 2000). In eukaryotes, cAMP and cGMP regulate enzyme and channel activity mainly through protein kinases, RapGEFs, or channels (Houslay and Milligan, 1997; Lohmann *et al.*, 1997; De Rooij *et al.*, 1998; Kraemer *et al.*, 2001). In addition to this large family of cAMP/cGMP binding proteins, some phosphodiesterases contain a GAF domain, which is an unrelated cGMP-binding domain that regulates enzyme activity (Francis *et al.*, 2000).

cAMP is probably present in all eukaryotes and cAMP-dependent protein kinase is a universal target even in primitive eukaryotes. Much less is known about the synthesis and function of cGMP in the lower eukaryotes. Yeast seems to lack cGMP, because the genome of *Saccharomyces cerevisiae* does not provide indications for putative guanylyl cyclases or cGMP-binding domains. Guanylyl cyclases have been identified in *Paramecium*, *Tetrahymena*, and *Plasmodium*, but the role of cGMP in these organisms is not yet resolved (Linder *et al.*, 1999; Carucci *et al.*, 2000).

In *Dictyostelium*, cAMP has an extracellular function as chemoattractant and an intracellular function as inducer of development (Reymond *et al.*, 1995). Extracellular cAMP binds to G protein-coupled receptors, which results in the activation of several signaling systems, including adenylyl cyclase, guanylyl cyclase, phosphatidylinositol 3-kinase, and calcium channels (Van Haastert and Kuwayama, 1997; Parent and Devreotes, 1999; Chung *et al.*, 2001). The produced intracellular cAMP is partly secreted where it activates neighboring cells. Intracellular cAMP may also bind to the regulatory subunit of cAMP-dependent protein kinase, mediating gene regulation and development. Eventually, cAMP is degraded by the extracellular phosphodiesterase PsdA (Lacombe *et al.*, 1986) and by the intracellular phosphodiesterase RegA (Shaulsky *et al.*, 1998; Thomason *et al.*, 1998).

Activation of the cAMP receptor also results in the transient activation of guanylyl cyclases. The produced cGMP is rapidly degraded, mainly by a cGMP-stimulated cGMP-specific phosphodiesterase (Ross and Newell, 1981; Van Haastert *et al.*, 1982b). As a consequence of the brief activa-

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tion of guanylyl cyclases and the substrate stimulation of phosphodiesterase activity, the cGMP accumulation has the shape of a spike with a maximum at 10 s and recovery of basal levels after 30 s. The function of cGMP in *Dictyostelium* probably concentrates on chemotaxis and osmoregulation, as was suggested by mutants defective in cGMP metabolism (Kuwayama *et al.*, 1993, 1996). Mutant *stm*F lacks the cGMP-stimulated phosphodiesterase (PDE) activity, whereas mutant KI8 shows very low levels of guanylyl cyclase activity. The genes defective in these mutants have not been identified.

To understand the function of cGMP in *Dictyostelium* it is essential to identify the genes that encode cGMP-metabolizing enzymes and cGMP target proteins. Recently, we characterized two unusual guanylyl cyclases in *Dictyostelium*, GCA and sGC, that are not related to vertebrate guanylyl cyclases, but are homologous to 12-transmembrane and soluble adenylyl cyclase, respectively (Roelofs *et al.*, 2001a,b). In addition, four genes were identified, named *gbpA-gbpD*, which possess putative cNB domains (Goldberg *et al.*, 2002). GbpC and GbpD are likely to mediate cGMP functions, because these proteins contain Ras, Kinase, and RasGEF domains besides the two putative cGMP-binding domains. Previous experiments have shown that *Dictyostelium* contains a cGMP-stimulated cGMP-phosphodiesterase (Van Haastert *et al.*, 1982a; Coukell *et al.*, 1984). We speculated that the cGMP-stimulated cGMP-phosphodiesterase is encoded by GbpA or GbpB, because these proteins contain a putative cGMP-binding domain and a $Zn^{2+}$-binding hydrolase domain that is distantly related to a small family of class II phosphodiesterases (Carfi *et al.*, 1995). We have inactivated the four *gbp* genes and analyzed the resulting cell lines for myosin phosphorylation and chemotaxis (Bosgraaf *et al.*, 2002). The experiments identified a cGMP-signaling cascade in which G protein-coupled receptors stimulate two novel guanylyl cyclases. The produced cGMP is transduced via GbpC to regulate myosin phosphorylation and assembly in the cytoskeleton, which are critical for chemotaxis. GbpA and GbpB were shown to be involved in the degradation of cGMP (Bosgraaf *et al.*, 2002). Herein, we report on the characterization of GbpA as the cGMP-stimulated cGMP-specific phosphodiesterase absent in mutant *stm*F, whereas GbpB seems to be a phosphodiesterase with dual specificity with respect to substrate and activation by both cAMP and cGMP.

## MATERIALS AND METHODS

### Strain and Culture Conditions

AX3 ("wild-type"), DH1 (an uracil auxotroph wild-type, kindly provided by P.N. Devreotes; Johns Hopkins Medical School, Baltimore, MD), and the mutant cell lines described below were grown in HG5 medium (HL5 with 10 g/l glucose) to a density of $\sim 2 \times 10^6$ cells/ml. When grown with selection, HG5 medium was supplemented with 10 $\mu$g/ml blasticidine S. Starved cells were obtained by shaking for 4–5 h in 10 mM phosphate buffer (PB), pH 6.5, at a density of $10^7$ cells/ml. Tight aggregates were obtained by starving the cells on nonnutrient agar for ~10 h; aggregates were collected in PB, washed by centrifugation, and disrupted to small cell clumps by passing the aggregates 10 times through a 0.5 × 16-mm needle.

### Gene Disruption

The disruptant strains were obtained as described previously (Bosgraaf *et al.*, 2002). Briefly, a 468-base pair genomic fragment of *gbpA* was obtained by polymerase chain reaction (PCR) by using primers TCATAGATCTAGAAGGTGATTATACAG and AGTTGGATCCATTGTTGCTAATTC. The PCR product was subcloned, and the Bsr selection cassette (Sutoh, 1993) was cloned into the MslI site of the genomic fragment. To disrupt the *gbpB* gene, a PCR product of 900 base pairs was amplified using the primers CCATTCTATGTGAAGTCAATC and AATTACTACTTACCAGCACC. The pyr5/6 cassette was cloned in the *Bcl*I restriction site. The selection cassette with *gbp* flanking sequences was amplified by PCR and ~5 $\mu$g of the PCR product was used to transform *Dictyostelium* DH1 cells. To select for transformants with the bsr cassette, HG5 was supplemented with 10 $\mu$g/ml blasticidin, whereas transformants with the pyr5/6 cassette were selected using uracil-deficient FM medium (Bio 101, Vista, CA). Potential knockouts were screened by PCR and confirmed by Southern analysis.

### Overexpression of GbpB in Dictyostelium

The full-length copy of *gbpB* without introns was obtained from cDNA fragments and PCR products. The *gbpB* sequence started with AGATCTAAAA**ATG**AATTCTAAATAT (the *Bgl*II restriction site underlined and the start codon in bold), whereas the sequences had a *Bam*HI restriction site engineered after the stop codon. The DNA was sequenced to verify the absence of mutations. The *Bgl*II/*Bam*HI fragment of full-length *gbpB* was cloned in the *Bgl*II site of plasmid AH2 and transformed to *gbpA*$^-$/*gbpB*$^-$ double-null cells. Plasmid AH2 is derivative of the extrachromosomal plasmid MB12neo (Heikoop *et al.*, 1998), except that the Neo selection and gene expression cassettes contain the actin8 terminator.

### Phosphodiesterase Assay of Dictyostelium Lysates

Cells were washed twice with PDE lysis buffer (40 mM HEPES/NaOH, pH 7.0, 0.5 mM EDTA) and resuspended at a density of $10^8$ cells/ml in PDE lysis buffer supplemented with 0.25 M sucrose. Cells were lysed by passage through a 0.45-$\mu$m Nuclepore filter. The lysate was centrifuged for 2 min at 14,000 × *g* and the supernatant was used.

The PDE assay mixture (final concentrations) contained assay buffer (40 mM HEPES/NaOH, pH 7.0, 0.5 mM EDTA, 0.25 M sucrose, 5 mM $MgCl_2$), 10 nM [$^3$H]cAMP, or 10 nM [$^3$H]cGMP as substrate, 5 mM dithiothreitol to inhibit the very active PDE1, and 30 $\mu$l of lysate in a total volume of 100 $\mu$l; the lysates were diluted to achieve between 10 and 30% hydrolysis of substrate. After incubation for 15 min at 22°C, reactions were terminated by boiling for 1 min. The product was dephosphorylated by calf intestine phosphatase (1 unit of enzyme in 100 $\mu$l of CIP buffer incubated for 1 h at 37°C). Finally, 300 $\mu$l of a 50% slurry of DOWEX AG1X2 was added to remove remaining substrate. After 15-min incubation at 22°C, samples were centrifuged for 2 min at 14,000 × *g*, and the radioactivity in 200 $\mu$l of the supernatant was determined.

### cAMP and cGMP Responses

Cells were starved for 5 h in PB, washed, and resuspended in PB to a density of $10^8$ cells/ml. For determination of the cGMP response, cells were stimulated with 0.1 $\mu$M cAMP and lysed at the times indicated by the addition of an equal volume of 3.5% (vol/vol) perchloric acid. Cells were stimulated with 10 $\mu$M 2′deoxy-cAMP and 10 mM dithiotreitol for induction of the cAMP response. Lysates were neutralized with $KHCO_3$, and cGMP and cAMP levels were determined by isotope dilution assays by using a cGMP-specific antibody or the regulatory subunit of cAMP-dependent protein kinase, respectively.

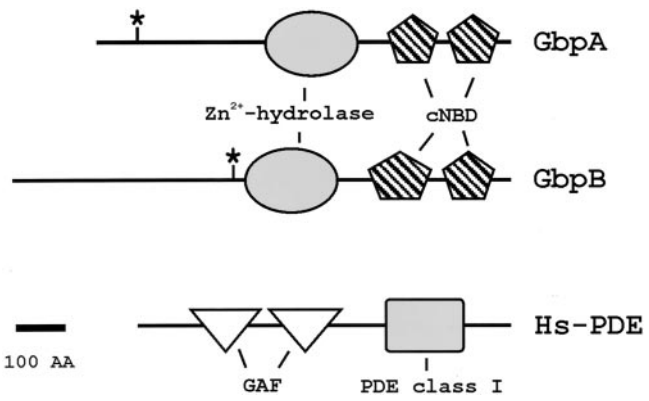


**Figure 1.** GbpA and GbpB. GbpA and GbpB have the same domain topology; a $Zn^{2+}$-hydrolase putative catalytic domain and two cNB domains. The asterisks indicate the position of disruption in the knockout cell lines. The topology of the cGMP-stimulated cAMP/cGMP phosphodiesterase from human (HsPDE) is shown for comparison; this enzyme has the same biochemical properties as GbpA, but is composed of a class I PDE domain and two cGMP-binding GAF domains.

### *Spore Formation*

The assay for induction of spore formation is essentially as described previously (Shaulsky *et al*., 1998; Thomason *et al*., 1998). Cells were washed and resuspended to a density of $4 \times 10^5$ cells/ml in spore buffer (10 mM MES, 10 mM NaCl, 10 mM KCl, 1 mM $CaCl_2$, 1 mM $MgSO_4$, pH 6.5), and 500 $\mu$l of the suspension was added to a well of a 24-well plate, yielding a density of $10^5$ cells/cm$^2$. Cells were incubated in the absence or presence of 5 mM cAMP or 20 $\mu$M Sp-cAMPS. After 36 h, when some spore-like cells were observed in some incubations, the buffer was replaced by PB with 0.5% (vol/vol) NP-40 to kill remaining amoebae. After 15 min at 22°C, samples were centrifuged for 3 min at $1000 \times g$, the pellet was washed twice with PB, and resuspended in 100 $\mu$l of PB. The number of viable spores was determined by plating 2 $\mu$l of the suspension in association with *Klepsiella aerogenes*. The number of colonies was determined three days later, and could be maximally 4000 if all amoebae were retrieved and converted to viable spores.

## RESULTS

### *Topology of GbpA and GbpB*

GbpA and GbpB are both composed of two potential cNB domains and one $Zn^{2+}$-binding domain (Figure 1). The alignment of the four cNB domains, together with the cNB domains of bacterial CAP protein, *Drosophila* protein kinase G (PKG), *Dictyostelium* protein kinase A (PKA), *Caenorhabditis elegans* cyclic nucleotide regulated channel, and Epac are presented in Figure 2A. The *Dictyostelium* cNB domains of GbpA and GbpB comply reasonably well with the consensus sequence, but are more divergent than for instance the cNB domains of *Dictyostelium* cAMP-dependent PKA. In the crystal structure of the CAP protein (Passner *et al*., 2000), cAMP interacts mainly with the amino acids IGEL and RSAxV (Figure 2A). In PKA, the amino acid at the position of the serine in RSA is an alanine, whereas in PKG this amino acid is a threonine and mutagenesis to alanine provides cAMP binding (Shabb *et al*., 1991). This region is

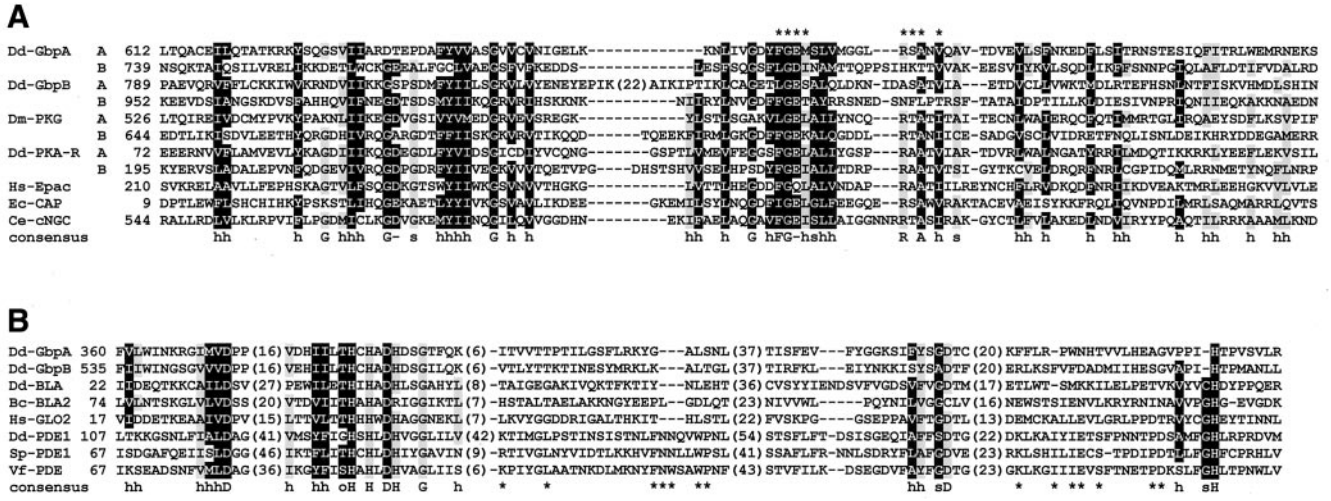


**Figure 2.** Sequence alignment. (A) Alignment of the four cNB domains from GbpA and GbpB with the cNB domains from *Drosophila* PKG, *Dictyostelium* regulatory subunit of PKA, human Epac, *Escherichia coli* CAP, and a *C. elegans* cyclic nucleotide-gated channel. The consensus is based on the alignment of 140 cNB domains; a black background indicates amino acids conserved in >80% of the sequences and a gray background in >60% of the sequences. The consensus sequence refers to hydrophobic (h), polar (o), small (s), and negatively charged (−) amino acids, or to specific amino acids (capital letters). The asterisks (*) denote the amino acids that have been shown to interact with cAMP in the CAP crystal structure (Passner *et al*., 2000). (B) Alignment of the putative $Zn^{2+}$-binding motifs of two $Zn^{2+}$-hydrolase domains from GbpA and GbpB with those from *Dictyostelium* β-lactamase (Dd-BLA), *Bacillus cereus* β-lactamase (Bc-BLA), human glyoxalase (Hs-GLO2), *Vibrio fischeri* phosphodiesterase (Vf-PDE), *Schizosaccharomyces pombe* phosphodiesterase (Sp-PDE), and *Dictyostelium* phosphodiesterase (Dd-PDE1). The consensus is based on 98 sequences with gray scales indicating conserved amino acids in >95% (black) or >80% (gray) of the sequences. The asterisks (*) indicates amino acids that are conserved in class II PDEs but are variant in GbpA and GbpB.

relatively poorly conserved in GbpA and GbpB, especially in the second cNB domains. The first cNB domains of both GbpA and GbpB possess a serine at the position of RTA, which may suggest that the first cNB domains more likely bind cGMP than cAMP. However, the cNB domains of GbpA and GbpB are also homologous to CAP proteins, which bind cAMP and cGMP with similar affinity and contain a serine at this position.

The $Zn^{2+}$-binding domains of GbpA and GbpB show a high degree of identity to each other (44% identity) and belong to the superfamily of β-lactamases with a metal-dependent hydrolase fold (Figure 2B). This domain is characterized by conserved histidines and aspartates that are also present in GbpA and GbpB. The superfamily of $Zn^{2+}$-binding domains contains many hydrolases such as β-lactamases, glyoxalases, and class II cyclic nucleotide phosphodiesterases (Carfi *et al.*, 1995). SMART and Pfam programs recognize the $Zn^{2+}$-binding domains of GbpA and GbpB as β-lactamases, but not as class II phosphodiesterases. The alignment reveals several amino acids that are conserved in class II phosphodiesterases, but not in GbpA and GbpB (Figure 2B, asterisks). Also phylogenetic analysis indicates that the $Zn^{2+}$-binding domains of GbpA and GbpB are more closely related to the β-lactamases than to the monophyletic group of class II phosphodiesterases (our unpublished data).

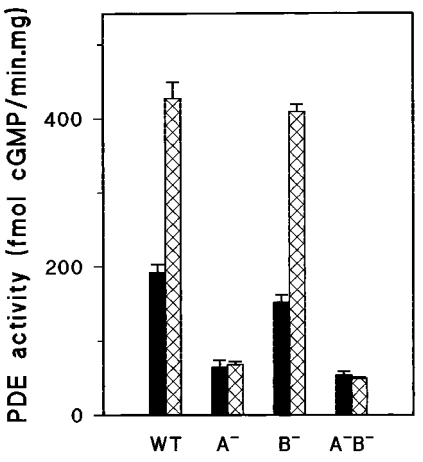


**Figure 3.** cGMP-PDE activity in vegetative *gbp*$^-$ null cells. The hydrolysis of 10 nM [3H]cGMP in the absence (black bar) or presence of 1 μM 8-bromo-cGMP (hatched bar) was measured in the supernatant of a lysate prepared from 2-h starved wild-type DH1 cells (WT), *gbpA*$^-$ cells (A$^-$), *gbpB*$^-$ cells (B$^-$), and the double-null *gbpA*$^-$/*gbpB*$^-$ cells (A$^-$B$^-$). The results shown are the means and SDs of three independent experiments with triplicate determinations and reveal a loss of 8-bromo-cGMP–stimulated phosphodiesterase activity in *gbpA*$^-$ cells.

### *GbpA Encodes a cGMP-stimulated cGMP-specific Phosphodiesterase*

To investigate the function of GbpA and GbpB, *Dictyostelium* cells were transformed with knockout constructs. Clones were screened by PCR for putative knockout strains and confirmed by Southern blots (data not shown). In this way, three cell lines were obtained with single and double knockouts of the *gbp* genes. The expression of *gbpA* and *gbpB* in knockout strains was investigated using Northern blots, demonstrating the absence of expression of even a truncated messenger in the knockout strains (data not shown).

The main cGMP-phosphodiesterase activity in *Dictyostelium* can be stimulated by the analog 8-bromo-cGMP (Van Haastert *et al.*, 1982b). To test whether the cGMP-stimulated cGMP-phosphodiesterase is encoded by *gbpA* and/or *gbpB*, we measured cGMP-phosphodiesterase activity in the absence and presence of 8-bromo-cGMP in the *gbp*$^-$ null strains. High levels of cGMP-PDE activity were found in wild-type cells, and this activity was stimulated two- to threefold by 8-bromo-cGMP (Figure 3). This enzyme activity was also present at high levels in *gbpB*$^-$ null cells, indicating that *gbpB* does not encode the enzyme. In contrast, cGMP-phosphodiesterase activity was very low in *gbpA*$^-$ null cells, and this small activity was not stimulated by 8-bromo-cGMP. The residual activity in *gbpA*$^-$ cells had the kinetic properties of DdPDE3 (low $K_M$ value and inhibition by isobutylmethylxanthine; our unpublished data; Kuwayama *et al.*, 2001). The double mutant *gbpA*$^-$/*gbpB*$^-$ had a similar low cGMP-phosphodiesterase activity as *gbpA*$^-$. These results indicate that *gbpA* encodes the well-characterized cGMP-stimulated cGMP-phosphodiesterase activity in *Dictyostelium* and that *gbpB* may encode a phosphodiesterase with different biochemical properties.

### *GbpB May Encode a Dual Specificity Phosphodiesterase Stimulated by cGMP and cAMP*

Northern blots reveal that *gbpB* is expressed maximally in the multicellular stage (Goldberg *et al.*, 2002). Therefore, we measured phosphodiesterase activity in lysates prepared from tight aggregates. Phosphodiesterase activity of *gbpA*$^-$ null cells is composed of several (partly unknown) phosphodiesterases except GbpA, whereas *gbpA*$^-$/*gbpB*$^-$ cells possess the same mixture of enzymes except GbpA and GbpB. Thus, by subtracting the activity of *gbpA*$^-$/*gbpB*$^-$ lysates from *gbpA*$^-$ lysates, information on GbpB is obtained. Similarly, the difference of enzyme activity between *gbpB*$^-$ and *gbpA*$^-$/*gbpB*$^-$ yields the biochemical properties of GbpA. Assays were conducted with [$^3$H]cAMP or [$^3$H]cGMP as substrate in the absence or presence of 8-bromo-cAMP or 8-bromo-cGMP as activators (Figure 4).

The lysates prepared from tight aggregates of *gbpA*$^-$/*gbpB*$^-$ double null cells contain a small cGMP- and cAMP-hydrolyzing activity that is not affected by 8-bromo-cAMP or 8-bromo-cGMP. GbpA is characterized by the additional activity in *gbpB*$^-$ cells, demonstrating cGMP-hydrolyzing activity that is stimulated fourfold by 8-bromo-cGMP; 8-bromo-cAMP has no effect, and cAMP is not a substrate. These deduced properties of GbpA in tight aggregates are essentially identical to those of GbpA in aggregation-competent cells described above. GbpB was characterized using *gbpA*$^-$ cells, showing a small cGMP- and a larger cAMP-hydrolyzing activity on top of the cGMP- and cAMP-hydrolyzing activity of *gbpA*$^-$/*gbpB*$^-$ double-null cells. This activity is stimulated by both 8-bromo-cAMP and 8-bromo-cGMP. These findings suggest that GbpB might be a dual specificity phosphodiesterase, both in respect to the substrate as well as the activator. However, the activity is rather low for a full

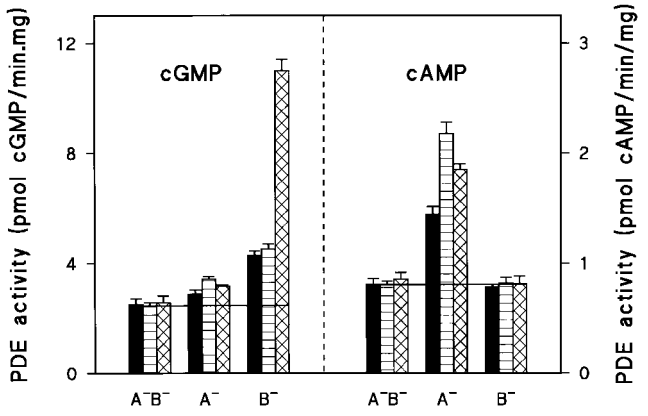


**Figure 4.** cGMP and cAMP PDE activity in tight aggregate *gbp*$^-$ null. Cells were starved and developed on nonnutrient agar until the tight aggregate stage, collected, dissociated, and lysed. The hydrolysis of 10 nM [$^3$H]cGMP or [$^3$H]cAMP was measured in the absence (black bar) or presence of 1 $\mu$M 8-bromo-cGMP (cross-hatched bar) or 8-bromo-cAMP (striped bar) in the supernatant of a lysate prepared from *gbpA*$^-$ cells (A$^-$), *gbpB*$^-$ cells (B$^-$), and the double-null *gbpA*$^-$/*gbpB*$^-$ cells (A$^-$B$^-$). The difference in activities between A$^-$B$^-$ and A$^-$ characterizes GbpB, whereas the difference between A$^-$B$^-$ and B$^-$ provides information of GbpA. The results confirm the observations in Figure 3 that GbpA encodes a cGMP-stimulated cGMP-PDE, and suggest that GbpB hydrolyzes cAMP and perhaps cGMP and is activated by both 8-bromo-cAMP and 8-bromo-cGMP.

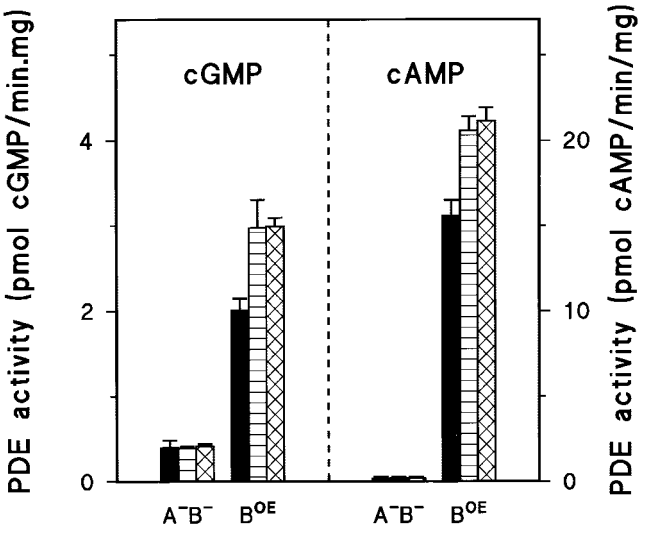


**Figure 5.** Overexpression of GbpB in *gbpA*$^-$/*gbpB*$^-$ cells The hydrolysis of 10 nM [$^3$H]cGMP or [$^3$H]cAMP was measured in the absence (black bar) or presence of 1 $\mu$M 8-bromo-cGMP (cross-hatched bar) or 8-bromo-cAMP (striped bar) in the supernatant of a lysate prepared from *gbpA*$^-$/*gbpB*$^-$ cells (A$^-$B$^-$) and from *gbpA*$^-$/*gbpB*$^-$/*GbpB*$^{OE}$ (B$^{OE}$). The results demonstrate that GbpB hydrolyses cAMP ~8-fold faster than cGMP; both 8-bromo-cAMP and 8-bromo-cGMP activate the enzyme.

biochemical characterization of GbpB, and therefore we overexpressed GbpB in *Dictyostelium*.

### *Overexpression of GbpB in Dictyostelium gbpA*$^-$*/gbpB*$^-$ *Cells*

GbpB was expressed in growing cells from a strong actin promoter by using the extrachromosomal expression vector AH2. We used the double null *gbpA*$^-$/*gbpB*$^-$ as host to have a null background of GbpA and GbpB enzyme activity. The lysates of *gbpA*$^-$/*gbpB*$^-$/GbpB$^{OE}$ cells contain cAMP- and cGMP-hydrolyzing activity that is much higher that the activity observed in lysates from *gbpA*$^-$/*gbpB*$^-$ cells (Figure 5). The increase of cAMP-hydrolyzing activity is 15 pmol/min/mg protein, which is ~60-fold higher than the estimated endogenous GbpB activity of wild-type cells at 10 nM cAMP (~0.26 pmol/min/mg protein; Table 2). Overexpression of GbpB provides a much smaller increase of cGMP-hydrolyzing activity (1.6 pmol/min/mg protein above the activity in *gbpA*$^-$/*gbpB*$^-$ cells), indicating that GbpB is ~9-fold more active toward cAMP than toward cGMP. Both the cAMP- and cGMP-hydrolyzing activity are stimulated ~1.5-fold by 1 $\mu$M 8-bromo-cGMP and 8-bromo-cAMP. These data on overexpressed GbpB confirm the provisional conclusions on phosphodiesterase activity in tight aggregates of *gbpA*$^-$ that GbpB is a dual-specificity phosphodiesterase with preference for cAMP.

### *Biochemical Properties of GbpA and GbpB*

The biochemical properties of GbpB were determined using the *gbpA*$^-$/*gbpB*$^-$/*GbpB*$^{OE}$ cells (Figure 6). The hydrolysis of 10 nM [$^3$H]cAMP or 10 nM [$^3$H]cGMP was measured in the absence or presence of different concentrations of cAMP or cGMP, respectively. Figure 6A demonstrates that low concentrations of cAMP stimulate the hydrolysis of 10 nM [$^3$H]cAMP, whereas concentrations above 10 $\mu$M cAMP inhibit the hydrolysis of [$^3$H]cAMP. For the hydrolysis of 10 nM [$^3$H]cGMP we observed similar properties: stimulation at low concentrations of cGMP and inhibition at high cGMP concentrations. These data were used to obtain the activation constant $K_A$, the Michaelis-Menten constant $K_M$, and the $V_{MAX}$ of GbpB. Figure 6B demonstrates that cAMP and cGMP stimulate the enzyme maximally 1.5- and 1.9-fold, respectively. The $K_A$ value is 0.71 $\mu$M for cAMP and 2.3 $\mu$M for cGMP. The data on the hydrolysis of cAMP and cGMP are presented as Eady-Hofstee plot in Figure 6C, demonstrating activation at low concentrations and linear curves at higher concentrations. The slopes of the linear parts yield a $K_M$ value of 200 $\mu$M for cAMP and 800 $\mu$M for cGMP, whereas the intercepts with the abscissa yield a $V_{MAX}$ value of 650 and 300 nmol/min/mg protein for cAMP and cGMP, respectively.

The biochemical properties of GbpA were derived from a partially purified enzyme from wild-type cells (Van Haastert and Van Lookeren Campagne, 1984) by using the same analysis as for GbpB. The enzyme preparation does not show hydrolysis of [$^3$H]cAMP, indicating that cAMP hydrolysis is at least 100-fold slower than cGMP. Figure 7A reveals that low concentrations of cGMP stimulate the hydrolysis of [$^3$H]cGMP, whereas concentrations above 1 $\mu$M inhibit the hydrolysis of [$^3$H]cGMP; cAMP does not activate the hydrolysis of [$^3$H]cGMP but inhibits at very high concentrations with a $K_I$ value of 1.8 mM. The activation constant $K_A$ of GbpA for cGMP is 0.16 $\mu$M, and the enzyme is activated

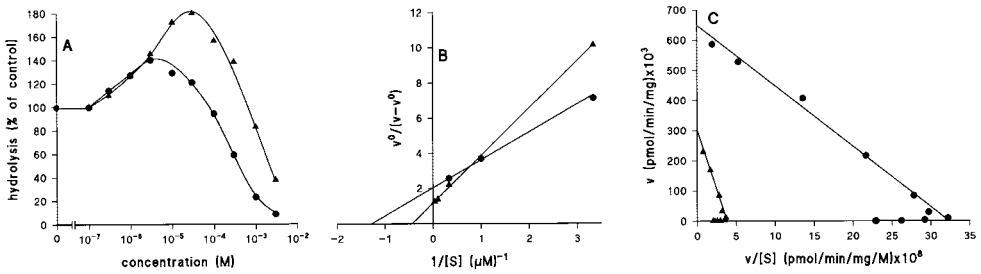


**Figure 6.** Characterization of GbpB. A lysate was prepared from *gbpA*$^-$/*gbpB*$^-$/*GbpB*$^{OE}$ cells and incubated with 10 nM [$^3$H]cGMP or [$^3$H]cAMP in the presence of different concentrations of unlabeled cGMP or cAMP, respectively. The effect of unlabeled cAMP (●) or cGMP (▲) on the hydrolysis of the radioactive tracer is presented in A. These data are converted in panel B (see Van Haastert and Van Lookeren Campagne, 1984); $v^o$ is the hydrolysis in the absence and v in the presence of the indicated concentrations of unlabeled substrate [S]. The plot allows the determination of the activation constant $K_A$ (intercept abscissa is $-1/K_A$) and the maximal fold activation $A_{MAX}$ (intercept ordinate is $1/(A_{MAX} - 1)$). The data of A were also used for an Eady-Hofstee plot (C) to calculate the $K_M$ value of the activated enzyme (slope is $-1/K_M$) and $V_{MAX}$ (intercept ordinate). The obtained kinetic data are listed in Table 2.

maximally 2.4-fold (Figure 7B). The Eady-Hofstee plot reveals a Michaelis-Menten constant $K_M$ value for cGMP of 5.2 $\mu$M.

In summary, GbpA and GbpB are novel cyclic nucleotide stimulated cyclic nucleotide phosphodiesterases. GbpA is a cGMP-specific enzyme, whereas GbpB is a dual specificity enzyme with preference for cAMP. Activation of GbpB occurs at higher cGMP concentrations than activation of GbpA and does not discriminate between cAMP and cGMP; in contrast, activation of GbpA is at least 300-fold more specific for cGMP than for cAMP.

### *cGMP Response in gbpA and gbpB Mutants*

The consequences of deletion of gbpA and gbpB on basal cGMP levels and on the cAMP-mediated cGMP response of 5-h starved cells are presented in Figure 8A. Basal cGMP levels in wild-type cells are ~1 pmol/10$^7$ cells that increase to 6 pmol/10$^7$ cells upon stimulation with cAMP; maximal levels are obtained after 10 s, and basal levels are recovered after ~30 s. Deletion of the cGMP-stimulated cGMP-PDE in *gbpA*$^-$ cells leads to an increase of basal cGMP levels from 1 to 3 pmol/10$^7$ cells. The cAMP-mediated cGMP response is enlarged from 6 to ~15 pmol/10$^7$ cells; the cGMP accumulation continues and persists for a longer period than in wild-type cells, causing the cGMP peak to occur at 20 s; basal levels are recovered after ~120 s. The altered cGMP response in *gbpA*$^-$ cells is essentially identical to the cGMP response in the mutant *stm*F, which also lacks the cGMP-stimulated cGMP-PDE (Van Haastert *et al.*, 1982a).

Disruption of *gbpB* has only a small effect on cGMP levels (Figure 8B); basal levels and the cGMP response are increased by ~25% relative to wild-type cells, confirming the relatively small contribution of GbpB to the total cGMP-PDE activity in vivo. The potential cGMP-hydrolyzing activity of GbpB can be demonstrated in a *gbpA*$^-$ null cells, which lack the major cGMP-PDE activity. Disruption of *gbpB* in a *gbpA*$^-$ background results in a further increase of basal cGMP levels from 3 pmol/10$^7$ cells in *gbpA*$^-$ to 12 pmol/10$^7$ cells in *gbpA*$^-$/*gbpB*$^-$. The cAMP-induced cGMP response is also substantially enhanced and prolonged from a maximum of 15 pmol/10$^7$cells at 20 s after stimulation in *gbpA*$^-$ to 40 pmol/10$^7$ cells at 30 s after stimulation in the *gbpA*$^-$/*gbpB*$^-$ strain; basal levels were reached after ~3–4 min (Figure 8A). These results demonstrate that the low cGMP-PDE activity of GbpB becomes functionally significant when the much more active cGMP-PDE activity of GbpA is deleted.

Overexpression of GbpB in *gbpA*$^-$/*gbpB*$^-$ cells whips out the dramatic cGMP response seen in the *gbpA*$^-$/*gbpB*$^-$ cells

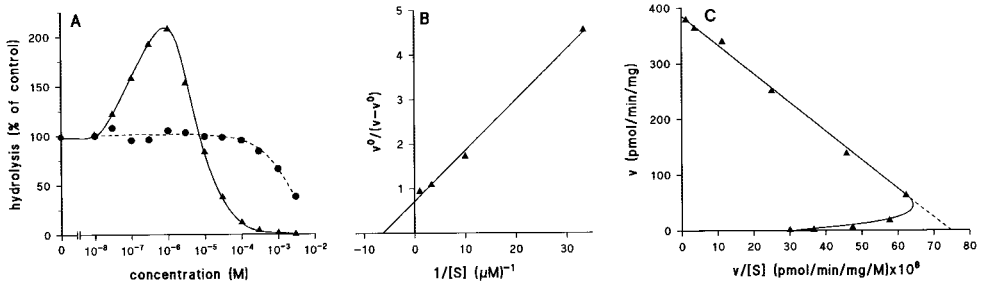


**Figure 7.** Characterization of GbpA. A partially purified preparation of GbpA was prepared from wild-type cells and incubated with 10 nM [$^3$H]cGMP in the presence of different concentrations of unlabeled cGMP (▲) or cAMP (●). The enzyme preparation does not show detectable hydrolysis of [$^3$H]cAMP, indicating that cAMP is hydrolyzed at least 100-fold slower than cGMP. The kinetic constants for cGMP were derived from the plots in B and C, as described in Figure 6, and are listed in Table 2.

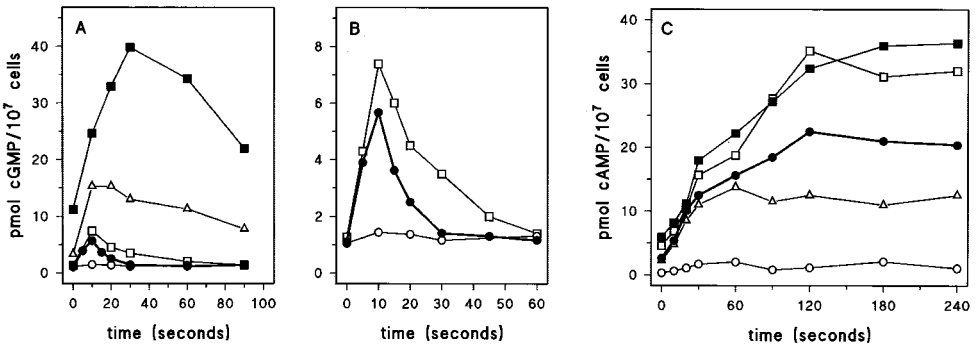


**Figure 8.** cGMP and cAMP responses in *gbp*$^-$ mutant cells. The cell were starved for 5 h followed by stimulation with 0.1 $\mu$M cAMP for the cGMP response (A and B) and with 10 $\mu$M 2′-deoxy-cAMP and 10 mM dithiotreitol for the cAMP response (C). The symbols refer to wild-type DH1 (●); *gbpA*$^-$ (△); *gbpB*$^-$ (□); *gbpA*$^-$/*gbpB*$^-$ (■), and *gbpA*$^-$/*gbpB*$^-$/*GbpB*$^{OE}$ (○). Identical data are presented for wild-type DH1, *gbpB*$^-$, and *gbpA*$^-$/*gbpB*$^-$/*GbpB*$^{OE}$ in A and B. The results shown are the means of triplicate determinations from a typical experiment repeated once. The data in panel A were derived, in part, from Bosgraaf *et al.* (2002).

(Figure 8B). Basal cGMP levels are ~1.1 pmol/$10^7$ cells in the overexpressor strain relative to 12 pmol/$10^7$ cells in the parental *gbpA*$^-$/*gbpB*$^-$ cells and 1 pmol/$10^7$ cells in wild-type cells. The cGMP response is also substantially reduced to 1.5 pmol/$10^7$ cells, which is even much lower than the cGMP response of wild-type cells.

### *cAMP Response in gbpA and gbpB Mutants*

Stimulation of aggregation-competent cells with cAMP induces a transient accumulation of intracellular cAMP. In wild-type cells ~50% of the produced cAMP is secreted and ~50% is degraded intracellularly (Dinauer *et al.*, 1980). We stimulated cells with 2′-deoxy-cAMP and dithiotreitol and measured the accumulation of cAMP in the cell suspension. The analog 2′-deoxy-cAMP binds to surface cAMP receptor with high affinity but does not interfere with the determination of cAMP levels. Dithiotreitol inhibits the surface and extracellular PDE activity encoded by the *psdA* gene, but has no effect on GbpA or GbpB (our unpublished data). Thus, in this experiment we detect receptor-stimulated cAMP formation that eventually accumulates in the extracellular medium; the data below are presented for $10^7$ cells. Basal cAMP levels of wild-type cells is ~2.6 ± 0.5 pmol (Figure 8C); 2′-deoxy-cAMP induces the accumulation of cAMP at an initial rate of ~0.35 ± 0.06 pmol/s, and the final increase of (extracellular) cAMP is 18.7 ± 1.1 pmol above basal levels. Deletion of the cAMP-PDE in *gbpB*$^-$ cells leads to an increase of basal cAMP levels to 4.6 ± 0.7 pmol. The 2′-deoxy-cAMP–mediated increase of cAMP levels shows approximately the same initial rate as in wild-type cells (0.38 ± 0.10 pmol/s), but continues for a longer period by which eventually ~1.6-fold more cAMP accumulates in the extracellular medium (28.2 ± 4.4 pmol). Basal cAMP levels and the cAMP response in *gbpA*$^-$/*gbpB*$^-$ cells are essentially identical to the response seen in *gbpB*$^-$ cells. The normal initial cAMP accumulation rate in *gbpB*$^-$ and *gbpA*$^-$/*gbpB*$^-$ cells strongly suggests that the receptor-stimulated production of cAMP is not altered in the mutants. The increased accumulation of extracellular cAMP indicates that, by deleting the cAMP-PDE activity of GbpB, intracellular cAMP is not effectively degraded and more cAMP is available for secretion. Because in wild-type cells ~50% of the produced cAMP is degraded intracellularly, complete inhibition of this degradation would induce not more than a twofold increase of the cAMP response.

GbpA does not hydrolyze cAMP, but may affect the cAMP response indirectly, because the enzyme regulates cGMP levels, and cGMP activates the cAMP-PDE activity of GbpB. Consistent with this notion, we observed that the extracellular cAMP accumulation in *gbpA*$^-$ cells is reduced ~50% relative to cAMP accumulation in wild-type cells. The initial cAMP accumulation rate is unaffected (0.33 ± 0.09 pmol/s), but the accumulation plateaus to a lower level (9.5 ± 1.1 pmol), suggesting that the same amount of cAMP is produced but less cAMP is available for secretion.

Overexpression of GbpB leads to a very strong reduction of the cAMP response, basal levels are decreased to 0.3 ± 0.2 pmol, ~12% from wild-type cells, and the cAMP accumulation is only 1.2 ± 0.2 pmol, which is only 6% of the response seen in wild-type cells. The results suggest that GbpB is an important PDE to modulate intracellular cAMP levels. Null cells show increased cAMP levels, whereas overexpression leads to a strong reduction of cAMP.

### *Phenotypes of gbpA and gbpB Mutants*

Cell aggregation of *gbpA*$^-$ cells, *gbpB*$^-$ cells, and *gbpA*$^-$/*gbpB*$^-$ cells is normal compared with wild-type cells (our unpublished data). The aggregation time is not different from wild-type cells, and fruiting bodies have a relatively normal size. Overexpression of GbpB (*gbpA*$^-$/*gbpB*$^-$/*gbpB*$^{OE}$ cells) leads to very slow and poor aggregation (Figure 9). Cell aggregation in wild type starts at 8 h and fruiting body formation is completed after ~20 h. The *gbpA*$^-$*B*$^-$/*gbpB*$^{OE}$ cells start to aggregate at ~12 h after the onset of starvation, and slugs are first visible after 15 h, which is at least 4 h later than in wild-type cells. Eventually, fruiting bodies are formed after 27 h, but many cells do not participate in multicellular development.

The phenotype of GbpB overexpression is similar to the phenotype of overexpression of RegA, the first characterized intracellular cAMP-PDE in *Dictyostelium* (Shaulsky *et al.*, 1998; Thomason *et al.*, 1998). Deletion of the *regA* gene has

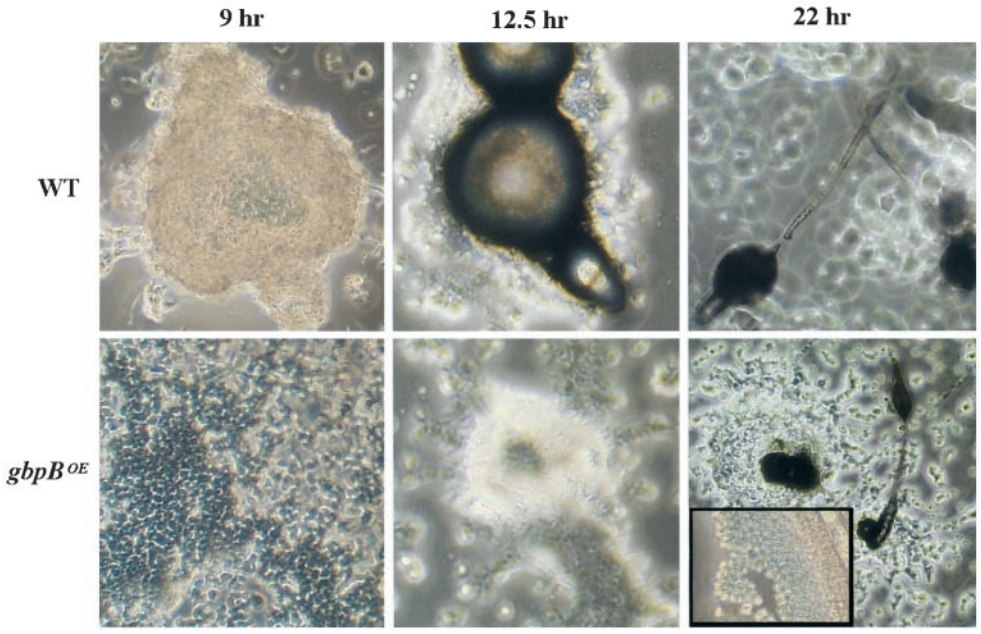


**Figure 9.** Phenotype of $GbpB^{OE}$ mutant cells. GbpB was overexpressed in $gbpA^-/gbpB^-$ cells reaching an activity that was ~50-fold higher than GbpB activity of wild-type cells. Photographs show the phenotypes at different times after starvation; the inset shows normal spore formation after 27 h in $gbpA^-/gbpB^-/gbpB^{OE}$ cells.

been shown to lead to increased intracellular cAMP levels, leading to a sporogenous phenotype: spores are formed in the multicellular stage earlier than in the wild type, and extracellular cAMP can induce spore formation in monolayers of $regA^-$ cells under buffer. We tested whether deletion of GbpB, the second cAMP-PDE in addition to regA, also leads to a sporogeneous phenotype (Table 1). Wild-type cells did not form spores when incubated with cAMP in submerged conditions. In contrast, a significant fraction of $gbpB^-$ and $gbpA^-/gbpB^-$ cells had form spores. This was not observed for the $gbpA^-$ cells, indicating that the sporogenous phenotype is specific for deletion of a cAMP-PDE activity.

## DISCUSSION

Two families of cyclic nucleotide phosphodiesterases have been recognized in eukaryotes, the ubiquitous class I phosphodiesterases present in essentially all eukaryotes and the small family of class II enzymes found in some bacteria, several yeast species, and *Dictyostelium* (the surface cAMP-phosphodiesterase PsdA). The class II enzymes belong to the superfamily of proteins with a $Zn^{2+}$-binding hydrolase fold that also includes β-lactamases, glyoxylases, and arylsulfatases (Carfi *et al.*, 1995). The GbpA and GbpB enzymes described in this report are phosphodiesterases and members of the superfamily, but sequence alignment and phylogeny suggest that they are not very closely related to the subfamily of class II phosphodiesterases (Goldberg *et al.*, 2002; our unpublished data). The domain programs SMART and Pfam support this notion, because they recognize GbpA and GbpB as β-lactamases, but not as class II phosphodiesterases.

**Table 1.** Spore formation in *gbp* mutants

| Strain | No. of viable spores | |
|---|---|---|
| Wild type | 0 | 0 |
| $gbpA^-$ | 0 | 1 |
| $gbpB^-$ | 28 | 47 |
| $gbpA^-/gbpB^-$ | 30 | 46 |

Cells were incubated under buffer with cAMP for 36 h, treated with 0.5% NP-40 to kill amoebae, and plated in association with bacteria. The figures refer to the number of colonies obtained in two experiments, each derived from the original 4000 amoebae.

Inactivation and overexpression of the *gbpA* and *gbpB* genes indicate that *gbpA* encodes the cGMP-stimulated cGMP-specific phosphodiesterase characterized previously at a biochemical level (Van Haastert and Van Lookeren Campagne, 1984), whereas *gbpB* encodes a novel cAMP/cGMP-stimulated dual-specificity enzyme. Using about 20 cGMP analogs to characterize GbpA, it was demonstrated that the cyclic nucleotide specificity for activation and hydrolysis are very different, which was regarded as strong evidence that the enzyme possesses different cGMP-binding sites for activation and catalysis (Kesbeke *et al.*, 1985). The domain structure of GbpA supports this hypothesis, because the enzyme is composed of a $Zn^{2+}$-binding hydrolase fold, likely mediating hydrolysis of cGMP, and two cNB domains of which one is predicted to be a cGMP-binding regulatory domain. The kinetic properties of GbpA are summarized in Table 2, showing half-maximal activation at 0.16 μM cGMP, and a $K_M$ value of 5–20 μM cGMP for the cGMP-activated

**Table 2.** Properties of six *Dictyostelium* PDEs

| Phosphodiesterase | PDE1 | PDE2 | PDE3 | PDE4* | PDE5 | PDE6 |
|---|---|---|---|---|---|---|
| Name of gene | *psdA* | regA | *DdPDE3* | *DdPDE4* | *gbpA* | *gbpB* |
| Localization | Cell surface | Cytosol | Cytosol | Cell surface | Cytosol | Cytosol |
| Class | II | I | I | I | II | II |
| cAMP/cGMP selectivity | 3 | >200 | ~0.0015 | Unknown | <0.003 | 9 |
| cAMP hydrolysis | | | | | | |
| $K_M$ ($\mu$M) | 0.8 | 5 | >100 (150) | Unknown | >500 (1800) | 200 |
| $V_{MAX}$ (pmol/min/mg) | 700 | 50 | – | Unknown | – | 5200 |
| $K_A$ ($\mu$M) | – | – | – | Unknown | >300 | 0.7 |
| $A_{MAX}$ | – | – | – | – | – | 1.47 |
| cGMP hydrolysis | | | | | | |
| $K_M$ ($\mu$M) | 1.8 | >1000 | 0.22 | –* | 5.2–20 | 800 |
| $V_{MAX}$ (pmol/min/mg) | 490 | – | 2 | –* | 390 | 2400 |
| $K_A$ ($\mu$M) | – | – | – | –* | 0.16 | 2.3 |
| $A_{MAX}$ | – | – | – | – | 2.40 | 1.86 |
| Intracellular cAMP degradation (pmol/min/mg) | | | | | | |
| 0.1 $\mu$M | – | 1 | – | –* | – | 2.6 |
| 1 $\mu$M | – | 8 | – | –* | – | 26 |
| 5 $\mu$M | – | 25 | – | –* | – | 127 |
| Intracellular cGMP degradation (pmol/min/mg) | | | | | | |
| 0.1 $\mu$M | – | – | 0.6 | –* | 3.3 | 0.3 |
| 1 $\mu$M | – | – | 1.6 | –* | 48 | 3.0 |
| 5 $\mu$M | – | – | 1.9 | –* | 163 | 15 |

PDE4*, the enzyme has not been characterized biochemically; sequence data suggest that the enzyme is cAMP specific and has a signal sequence and two transmembrane segments predicting the catalytic domain to be extracellular. The cAMP/cGMP selectivity refers to the calculated $V_{MAX}/K_M$ for cAMP divided by the $V_{MAX}/K_M$ for cGMP. The data for the $K_M$ in parentheses refer to the cAMP concentration inducing half-maximal inhibition of the hydrolysis of cGMP. The kinetic constants were derived from Van Haastert *et al.* (1983) for PDE1; from Shaulsky *et al.* (1998) and Thomason *et al.* (1998) for PDE2, and from Figure 4 to calculate the $V_{MAX}$ in vivo, assuming that cAMP hydrolysis in *gbpA*$^-$/*gbpB*$^-$ is derived from RegA; for PDE6 from Kuwayama *et al.* (2001) for PDE3; from Figure 7 for PDE5; and from Figure 6 for PDE6. The rates of intracellular degradation were calculated using the obtained kinetic constants and are presented for three concentrations representing basal levels (0.1 $\mu$M), maximal levels in wild-type cells (1 $\mu$M), and maximal levels in some deletion mutants (5 $\mu$M).

and -nonactivated enzyme, respectively. GbpA does not hydrolyze cAMP and is not stimulated by cAMP.

The biochemical phenotype of the *gbpA*$^-$ cells is very similar to that of the chemically mutated *Dictyostelium stm*F cell line, which both lacks the same cGMP-stimulated PDE activity (Ross and Newell, 1981; Van Haastert *et al.*, 1982b). Two alleles of *stm*F are known, NP368 that lacks all GbpA-PDE activity, and NP377 that shows ~5% of wild-type activity with altered $K_M$ for cGMP and altered $K_A$ for 8-bromo-cGMP (Van Haastert *et al.*, 1982b; Coukell and Cameron, 1986). Therefore, we expected a severe mutation in NP368 leading to the absence of GbpA-PDE activity and a more subtle mutation in the open reading frame of NP377, leading to reduced and altered activity. Unexpectedly, we and Meima *et al.* (2002) have not been able to identify a DNA mutation in the *gbpA* gene of NP368 and NP377, respectively. NP368 shows normal mRNA levels for *gbpA*. The 5′-untranslated region of *gbpA* from NP368 was cloned between the actin promotor and GFP and did not reduce the expression of green fluorescent protein. The complete genomic copy of *gbpA* from NP368 was amplified and sequenced but did not reveal a mutation that would lead to inactivation of the expressed enzyme (such as stop codons or mutations in the proposed metal-binding catalytic site). We observed a Gly-to-Asp mutation at position 69, far before the proposed catalytic site; at this position the corresponding GbpB sequence has an Asp (L.B., H.R., and P.V.H., unpublished observations). Meima *et al.* (2002) observed reduced *gbpA* transcript levels in NP377 but could not detect any mutations in the promoter sequence.

The *stm*F mutants were originally isolated as "streamers," making large streams of aggregating cells. However, revertants of the streamer phenotype have been shown still to be defective in cGMP-PDE activity, indicating that the streamer properties of *stm*F can be segregated from its altered cGMP-PDE activity (Coukell and Cameron, 1986). Consistent with this genetic analysis we did not observe a streamer phenotype in the cGMP-PDE–defective *gbpA*$^-$ cells. *Stm*F mutants show an altered chemotaxis response during cell aggregation (Ross and Newell, 1981). However, wild-type cells mixed with a large portion of *stm*F mutant cells chemotax as mutant cells, whereas *stm*F cells mixed with a large portion of wild-type cells behave essentially as wild-type cells (Chandrasekhar *et al.*, 1995). This suggests that the altered aggregation behavior of *stm*F is due to an altered chemotaxis signal rather than to a modified chemotaxis response. The reduced cAMP relay in *gbpA*$^-$ cells could explain this altered aggregation of *stm*F mutants.

GbpB is characterized as a dual-specificity enzyme with preference for cAMP; the enzyme is half-maximally activated by 2.3 $\mu$M cGMP and 0.7 $\mu$M cAMP, and hydrolyses cGMP ~9-fold slower than cAMP. The catalytic site of GbpA shows high affinity and high selectivity for cGMP ($K_M$ = 5 $\mu$M cGMP and $K_I$ = 1800 $\mu$M cAMP), whereas GbpB has a much lower affinity and selectivity ($K_M$ = 800 $\mu$M cGMP and 200 $\mu$M cAMP). Experimental observations with cGMP analogs have demonstrated that cGMP is bound in the catalytic site of GbpA through a hydrogen bond to $C^6O$, which cannot be formed with cAMP (Kesbeke *et al.*, 1985). It is conceivable that this hydrogen bond potential is absent in GbpB by which the affinity for cGMP is low and no strong discrimination between cAMP and cGMP is possible in the catalytic site. Similar differences in the activator sites of GbpA and GbpB may explain the high affinity and selectivity of GbpA for cGMP relative to the nonspecific activation of GbpB.

GbpA and GbpB are the fifth and sixth PDE enzymes cloned in *Dictyostelium*. Therefore, these proteins may also be addressed as DdPDE5 and DdPDE6, respectively[1]. The *Dictyostelium* genome has been sequenced to >97% completion, suggesting that these six genes encode all phosphodiesterases in *Dictyostelium*, and we can begin with a detailed analysis of the relative contribution and function of the enzymes in modulating cAMP and cGMP levels in *Dictyostelium* (Table 2). PDE1, encoded by the *psdA* gene, is a class II nonselective enzyme located on the cell surface and in the extracellular medium (Lacombe *et al.*, 1986). PDE2, encoded by the *regA* gene, is a cAMP-specific class I phosphodiesterase. The enzyme is located in the cytosol and is regulated by a histidine kinase and cAMP-dependent protein kinase (Shaulsky *et al.*, 1998; Thomason *et al.*, 1998). PDE3 is a high-affinity, cGMP-specific enzyme located in the cytosol (Kuwayama *et al.*, 2001). PDE4 has not been characterized biochemically, but the primary sequence predicts the enzyme to be cAMP specific; furthermore, a putative signal sequence and two transmembrane segments are strongly indicated by structure prediction programs, suggesting that the enzyme is located at the plasma membrane with the catalytic domain in the extracellular medium (S.B. and P.V.H., unpublished observations). The six phosphodiesterases can be divided in three class I enzymes (PDE2, 3, and 4) and three class II enzymes (PDE1, 5, and 6). It is intriguing that GbpA shows similar biochemical properties as mammalian cGMP-stimulated phosphodiesterase, although the protein sequences are completely different. The catalytic domain of mammalian cGMP-stimulated phosphodiesterase belongs to the large family of PDE class I enzymes, and the cGMP-binding regulatory domain is unrelated to the cNB domain of GbpA but belongs to the group of GAF domains (Francis *et al.*, 2000).

[1] The four previously recognized PDEs in *Dictyostelium* are as follows: PDE1, the class II enzyme encoded by the *psdA* gene (Lacombe *et al.*, 1986); PDE2, the class I cAMP-specific enzyme encoded by the *regA* gene (Shaulsky *et al.*, 1998; Thomason *et al.*, 1998); PDE3, the class I cGMP-specific enzyme (Kuwayama *et al.*, 2001); and PDE4, a sequence recognized in the database (clone JAX4b25f06.r1) coding for a class I putative phosphodiesterase; the cGMP-stimulated cGMP-PDE activity is not affected by disruption of the PDE4 gene (S.B. and P.V.H., unpublished data).

The cellular localization and cAMP/cGMP specificity of the six *Dictyostelium* phosphodiesterases suggest three functional groups: degradation of extracellular cAMP by PDE1 and PDE4, degradation of intracellular cAMP by PDE2 and PDE6, and degradation of intracellular cGMP by PDE3, PDE5, and PDE6. The estimated activities toward these substrates in vivo may provide information on the relative importance and functions of these enzymes. Because the biochemical properties of PDE4 have not been determined yet, the contribution of PDE4 in degradation of extracellular cAMP is unknown.

Intracellular cAMP is degraded by the basal activity of PDE2/RegA and PDE6/GbpB at approximately equal rates, suggesting that both enzymes are important. This hypothesis is supported by the observation that both *regA*$^-$ and *gbpB*$^-$ null cells are sporogenous. The finding that RegA is activated by cAMP-dependent protein kinase in vitro allows strong modulation of RegA phosphodiesterase activity by intracellular cAMP in vivo. Such modulation is also predicted for GbpB, which is activated by cAMP binding to its activating cNB domain. The *regA*$^-$ null cells may have a stronger phenotype than *gbpB*$^-$ null cells: *regA*$^-$ aggregates form multiple tips, whereas *gbpB*$^-$ aggregates are as in wild-type, and cAMP induces spore formation in ~10% of *regA*$^-$ null cells vs. ~1% of *gbpB*$^-$ null cells. It is conceivable that in vivo the activation of RegA by histidine kinase and cAMP-dependent protein kinase is stronger than the activation of GbpB by cAMP. In conclusion, intracellular cAMP is degraded by two complex phosphodiesterases that belong to different classes of enzymes and show entirely different mechanisms of regulation by cAMP.

Three enzymes participate in the degradation of intracellular cGMP. The relative affinities and capacities clearly demonstrate that PDE5/GbpA is the major cGMP-degrading enzyme in vivo. The high affinity but low capacity of PDE3 predicts that this enzyme mainly participates in modulating low cGMP concentrations. In agreement with this notion, we observed previously that PDE3 activity affects basal cGMP levels but does not contribute much to the degradation of the high cGMP levels that arise during stimulation (Kuwayama *et al.*, 2001). The cGMP-PDE activity of PDE6/GbpB is also much smaller than the cGMP-PDE activity of PDE5/GbpA. These relative cGMP-PDE activities easily explain the effect of deletions of the three enzymes on basal cGMP levels. Single knockouts of the small PDE3 and PDE6 activities have little effect, whereas inactivation of the high PDE5/GbpA activity strongly affects cGMP levels. In a background of *gbpA*$^-$ cells, PDE3 and PDE6 are the only enzymes degrading cGMP, and have approximately equal activity. Therefore, deletion of *gbpB* in a *gbpA*$^-$ null background strongly increases cGMP levels. The function of cGMP is closely associated with chemotaxis via regulation of myosin II phosphorylation and myosin filament formation (de la Roche and Cote, 2001). We have elaborated on a large study toward the function of cGMP in myosin II regulation and chemotaxis by using cGMP phosphodiesterase mutants, also including double knockouts of the two guanylyl cyclases GCA and sGC and double knockouts of the two cGMP targets proteins GbpC and GbpD (Bosgraaf *et al.*, 2002). The results demonstrate enhanced myosin II phosphorylation and filament formation in the *gbpA*$^-$/*gbpB*$^-$ mutant with elevated cGMP levels; this increased myosin phosphoryla-

tion is associated with improved chemotaxis due to the suppression of lateral pseudopodia. This phenotype of the *gbpA*$^{-}$/*gbpB*$^{-}$ mutant is consistent with the myosin and chemotaxis phenotype of mutant *stm*F that also has elevated cGMP levels (Liu and Newell, 1993; de la Roche and Cote, 2001).

In conclusion, we have identified novel cGMP- and cAMP-regulated phosphodiesterases with a combination of $Zn^{2+}$-hydrolase and cNB domains not observed before. GbpA is a cGMP-stimulated cGMP-specific phosphodiesterase modulating cGMP levels, whereas GbpB is a dual-specificity phosphodiesterase with preference for cAMP modulating intracellular cAMP levels involved in multicellular development.

## ACKNOWLEDGMENTS

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